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Interaction between Ouabain and the Phosphorylated Intermediate of Na,K-ATPase

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SUMMARY

After phosphorylation of electric eel Na,K-ATPase by Na^+, Mg^{2+}, and ATP was terminated by removing the unbound Mg^{2+}, the phosphoenzyme was able to bind ouabain upon the addition of 2 mM ouabain under certain conditions. This binding was demonstrated by a 50% inhibition of ATPase after the removal of unbound ouabain by a Sephadex G-50 column (ouabain trapping method). At 4°, this ouabain binding was observed on the K^+-sensitive phosphoprotein (E_2P) formed in the presence of 10 mM Na^+ but was not observed on the ADP-sensitive phosphoprotein (E_1P) formed in the presence of 1000 mM Na^+. The increase in the dephosphorylation rate of E_2P with various concentrations of K^+ paralleled the decrease in inhibition by the addition of 2 mM ouabain after the termination of phosphorylation. In 50–200 mM Na^+, the eel enzyme used here formed the E_1P-rich phosphoprotein, but this phosphoprotein could bind with ouabain, even though the presence of ADP or oligomycin, which prevents the conversion of E_1P to E_2P, partially interfered with this ouabain binding. At 25°, ouabain binding with E_1P-rich phosphoprotein was observed in higher yield (up to 71%), but in each of these cases ADP or oligomycin strongly inhibited ouabain binding. Moreover, ouabain binding with E_2P-rich phosphoprotein did not significantly change with temperature, but ouabain binding with E_1P-rich phosphoprotein increased more than 6 times at temperatures from 4° to 25°. From these results, it can be concluded that E_2P can bind with ouabain in the absence of free Mg^{2+} whereas E_1P cannot, and that the interconversion between E_1P and E_2P can be stimulated with ouabain binding and accelerated with elevation of temperature. ADP- and K^+-insensitive phosphoprotein probably is only a minor intermediate for ouabain binding.

INTRODUCTION

Na,K-ATPase is inhibited specifically by cardiac steroid or cardiac glycoside, and it is believed to be the receptor of these cardioactive drugs. Experiments using the radioactive cardiac glycosides ouabain and digitoxin have shown that certain ligands—the two most effective systems are Na^+-Mg^{2+}-ATP and Mg^{2+}-P_i—are necessary for the binding of Na,K-ATPase with cardiac glycosides. It has been suggested that the phosphorylated forms of Na,K-ATPase are the active forms for binding with cardiac glycosides. Binding in the Na^+-Mg^{2+}-ATP system probably occurs *in vivo*, since free Na^+ inhibits the binding of ouabain to Na,K-ATPase in the Mg^{2+}-P_i system (1).

Several different phosphorylated forms are found in the Na^+-Mg^{2+}-ATPase system. Among these, E_1P[1] and E_2P are believed to be the main phosphorylated intermediates of Na,K-ATPase. Sen *et al.* (2) presented indirect evidence that E_2P binds with ouabain in the absence of free Mg^{2+} and that E_1P may not bind with ouabain. Since brain and kidney Na,K-ATPase preparations form mainly E_2P as the phosphorylated intermediate, *N*-ethylmaleimide-treated enzyme, which is thought to form mainly E_1P, was used to determine whether E_1P binds with ouabain, but the results obtained were confusing (3–5). Furthermore, Wallick *et al.* (6) reported that the interaction of *N*-ethylmaleimide and Na,K-ATPase is a complex reaction involving formation of several different modified enzymes.

Besides these two phosphorylated forms of Na,K-ATPase, two other phosphorylated forms of this enzyme were found to bind with ouabain (7). One phosphorylated intermediate forms from ATP in the presence of low concentrations of Na^+ and high concentrations of Mg^{2+}, whereas the other forms in the presence of Mg^{2+} and P_i. The dephosphorylations of these two phosphoenzymes were not accelerated by either K^+ or ADP.

The electric eel enzyme forms the E_1P-rich phosphoen-

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[1] The abbreviations used are: E_1P, ADP-sensitive phosphorylated form of Na,K-ATPase; E_2P, K^+-sensitive phosphorylated form of Na,K-ATPase; CDTA, 1.2-cyclohexylene dinitrilotetraacetic acid.

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zyme as shown in the preceding paper (8), and here we have studied the interaction between the phosphorylated form of the electric eel enzyme and ouabain in order to determine the active form for ouabain binding.

The study of binding between ouabain and the phosphorylated enzyme reported by Sen *et al.* (2) is based on the stabilization of the phosphorylated form by ouabain. It is difficult to arrive at a conclusion using this method, if the active phosphorylated intermediate for ouabain binding is mixed with the inactive phosphorylated form. For our research, we developed another method, the ouabain trapping method, for identifying the active intermediate for ouabain binding. The basic principle behind this method is as follows: The amount of the phosphorylated active intermediate for ouabain binding might be reduced rapidly when its formation is stopped. If ouabain is added to such a system, the reaction of ouabain with this phosphorylated active intermediate (E*P) follows the parallel first-order reaction mechanism as follows:

$$E^*P \xrightarrow{k} E + P_i$$
$$[\text{Ouabain}] \xrightarrow{k'} \text{Ouabain-EP}$$

where k is the dephosphorylation rate constant and k' is the second-order binding rate constant of ouabain to the E*P. In this case, the final amount of the ouabain-enzyme complex is given by $\alpha/(1 + k/k'[\text{ouabain}])$, where α is the amount of E*P at the starting point of ouabain binding if dissociation of the ouabain-enzyme complex is negligible. Then, if $k'[\text{ouabain}] \geq \frac{1}{4}k$ and α is one-half of the total phosphoprotein, 10% or more inhibition of the enzyme would be expected after the perfect decay of the transient active form.

The following data support the validity of this hypothesis: (a) The binding of ouabain with the sugar site is very stable (9). (b) The association rate constant of ouabain with eel enzyme is 0.41 μM^{-1} min^{-1} at 30° (9) and that with bovine brain enzyme is 0.64 μM^{-1} min^{-1} at 25° (10), but the dephosphorylation rate constant of eel or kidney Na,K-ATPase is 240 min^{-1} at 21° (11) or 164 min^{-1} at 25° (12). (c) Our new electric eel enzyme can pass through Sephadex columns without any significant loss in enzyme activity or amount of protein, and the unbound ouabain can be completely removed from the enzyme by this gel filtration (13). Therefore, more than 0.2 mM ouabain can bind with the active form of the enzyme to some extent and its binding can be identified by determining the inhibition, even though the new formation of active intermediate is stopped.

MATERIALS AND METHODS

The enzyme used here was derived from the same Na,K-ATPase-rich membrane fragments of the electric organ of the electric eel as described in the preceding paper (8). Fluorescamine and oligomycin were obtained from Pierce Chemical Company (Rockford, Ill.) and Sigma Chemical Company (St. Louis, Mo.), respectively.

Ouabain trapping method (binding between ouabain and phosphorylated enzyme). Phosphorylation was performed in a 1-ml volume with 0.1 mM unlabeled ATP for 1 sec as described in the preceding paper (8). Phosphorylation was terminated and ouabain binding was started by the addition of 0.5 ml of a mixture containing 150 mM CDTA (pH 7.6), 6 mM ouabain, and the same concentration of Na^+ as in the phosphorylation medium. In some cases, K^+ or ADP was also added to this mixture. After 6 sec, the ouabain binding reaction was quenched by the addition of 0.2 ml of a mixture containing 200 mM KCl and 25 mM ADP. The addition of K^+ also stabilized the enzyme-ouabain complex (1, 10). The final concentrations of K^+ and ADP were about 24 mM and 3.1 mM, respectively. Immediately after this quenching, the reaction mixture was placed on a Sephadex G-50 column (1.5 cm × 30 cm) and eluted with 20 mM sodium borate buffer (pH 7.6). Suitable aliquots from the void volume were assayed for ATPase activity and protein content. This reaction procedure was performed by using the semi-rapid mixing apparatus described in the preceding paper (8).

When the ouabain concentration was 2 mM, the inhibition reached a steady level after 6 sec (10 mM Na^+, 4°), 1 sec (10 mM Na^+, 25°; see Fig. 5), or 3 sec (1000 mM Na^+, 25°; see Fig. 5). Then, finally, a 6-sec reaction time was chosen. As described under Introduction, the higher concentration of ouabain may inhibit more ATPase by this ouabain trapping method. However, because of the solubility of ouabain, 6 mM ouabain in the CDTA solution (the final ouabain concentration was 2 mM) was used here.

Assay methods. The assay for ATPase activity was performed at 30° by the linked pyruvate kinase-lactate dehydrogenase spectrophotometric method (14), and ATPase activity was calculated from duplicate assays. The protein content was assayed by the fluorescamine method (15). After a 0.1-ml aliquot containing 0.1–0.3 μg of protein in 20 mM sodium borate buffer (pH 7.6) was mixed with 0.9 ml of 0.2 M borax solution, 0.3 ml of fluorescamine solution (20 mg/100 ml of acetone) was added to the mixture while vortexing. The fluorescence was measured by a Farrand Ratio fluorometer, using a CS 7-51 Corning filter for the primary light source and a Corning CS 3-72 cutoff filter as the secondary source.

The phosphorylated enzyme was assayed as described in the preceding paper (8).

RESULTS

Interaction of phosphorylated Na,K-ATPase and ouabain at 4°. As shown in Table 1, the interaction of phosphoenzyme and ouabain was examined under various phosphorylation conditions, and inhibition by ouabain (ouabain binding) was observed only when the enzyme was phosphorylated. Moreover, the presence of high concentrations of Na^+ seemed to prevent this inhibition. These results suggest that the phosphoprotein formed in the presence of low concentrations of Na^+, namely E_2P, may bind with ouabain as reported by Sen *et al.* (2).

To confirm their idea, and to clarify whether the E_1P can bind ouabain, we studied the effects of Na^+, K^+, ADP, and oligomycin. As shown in Table 2, the addition of K^+ to the CDTA solution prevented ouabain binding at 4°. The addition of ADP did not affect it significantly

TABLE 1

Inhibition by the addition of ouabain after termination of Na,K-ATPase phosphorylation

The eel enzyme (about 0.2 mg) in 40 mM Tris-HCl buffer (pH 7.6) was phosphorylated and allowed to interact with ouabain by the series addition of ligands as indicated at 4°. The interval between each addition was 1 sec. The concentrations of the ligands during the phosphorylation were 100 mM Na^+, 2 mM Mg^{2+}, 0.1 mM ATP, and 40 mM Tris-HCl buffer (pH 7.6) unless otherwise indicated. The concentrations of CDTA and ouabain were 50 mM and 1 mM. This ouabain interaction was terminated by the addition of 60 mM KCl after 6 sec. The unbound ouabain was removed immediately by a Sephadex G-50 column, and inhibition of Na,K-ATPase was measured (see text).

Order of addition to enzyme for phosphorylation				Inhibition by ouabain
1st	2nd	3rd	4th	
				%
Na^+	Mg^{2+}	ATP	CDTA + ouabain	37
Na^+	Mg^{2+}	ATP + CDTA + ouabain	—	0
Na^+	Mg^{2+}	CDTA + ouabain	—	0
Na^+	ATP	CDTA + ouabain	—	3
Mg^{2+}	ATP	CDTA + ouabain	—	−5
Na^+, 1000 mM	Mg^{2+}	ATP	CDTA + ouabain	5
Na^+	Mg^{2+}	ATP	Na^+, 1000 mM, + CDTA + ouabain	10

in the presence of 10 mM or 1000 mM Na^+, but in the presence of 50 mM or 100 mM Na^+, that addition did to some extent. The effects of Na^+ concentration were also examined in the absence or presence of oligomycin (Fig. 1). In the absence of oligomycin, the increase in Na^+ concentration from 50 to 200 mM apparently increased ouabain binding, and oligomycin reduced it in this range of Na^+ concentration.

TABLE 2

Inhibition by the interaction between the phosphoenzyme and ouabain at 4°

The enzyme was phosphorylated for 1 sec at 4° by ATP in the presence of Na^+, 2 mM Mg^{2+}, and 40 mM Tris buffer (pH 7.6). This phosphorylation was terminated and ouabain binding was started by the addition of a CDTA-ouabain solution (pH 7.6 with Tris) containing the same concentration of Na^+ as in the phosphorylation. The final concentrations of CDTA and ouabain were 50 mM and 2 mM, respectively. K^+ or ADP was also present in this solution at the concentrations indicated. After 6 sec, interaction with the ouabain was quenched by the addition of 24 mM KCl and 3 mM ADP. Immediately after this quenching, the reaction mixture was applied to a Sephadex column (1.5 cm × 30 cm) and eluted with 20 mM sodium borate buffer (pH 7.6). ATPase activity and protein content were estimated in aliquots eluted at the void volume by the linked pyruvate kinase-lactate dehydrogenase spectroscopic method and the fluorescamine method, respectively.

Na^+	ATP during phosphorylation	Inhibition by ouabain		
		Without K^+, without ADP	With 2 mM K^+	With 1 mM ADP
mM	*μM*	%	%	%
10	20	49 ± 2	0.3 (6.3)[a]	43
50	20	62	1.5	41
100	20	64 ± 1	0	42 ± 1
100	100	63	1.0	—
1000	100[b]	12 ± 2	2.0 ± 0.5 (<0.5)[c]	10 ± 2

[a] 1 mM K^+ was used.
[b] 10 mM Mg^{2+} was used instead of 2 mM.
[c] 10 mM K^+ was used.

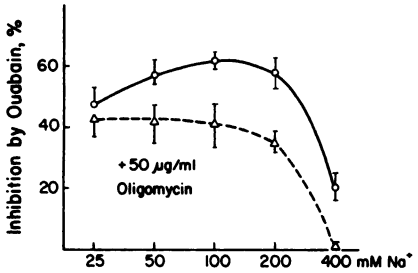


FIG. 1. *Effect of Na^+ concentration on inhibition by ouabain-phosphoprotein interaction at 4°*

The experimental procedures were similar to those described in Table 2, with the following exceptions: Phosphorylation was carried out by using 0.1 mM ATP, and ouabain binding was started by the addition of a Na^+-CDTA-ouabain solution with (Δ, *dashed line*) or without (○, *solid line*) oligomycin (50 μg/ml). Average values and standard deviations from triplet experiments are shown.

Comparison of the dephosphorylation rate with the decay rate of the active intermediate for ouabain binding. The formation of ADP- and K^+-insensitive phosphoenzyme in the presence of low Na^+ concentration and its binding with ouabain was reported by Post *et al.* (7). To compare the role of this insensitive phosphoprotein with that of E_2P in ouabain binding, the dephosphorylation rate was compared with the decay rate of the active intermediate for ouabain binding in the presence of various concentrations of K^+ (Fig. 2). The dephosphorylation rate constants coincided with the decay rate constants of the active intermediate in each reaction condition including the presence of ADP and K^+ (Table 3).

Effects of temperature on ouabain binding to phosphoenzyme. Temperature influenced inhibition by the ouabain trapping method (Fig. 3). In the presence of 10 mM Na^+, the temperature effect was not significant, but in the presence of 1000 mM Na^+, the inhibition was maximal at 25°. This maximal value in the presence of 1000 mM Na^+ was 6 times the inhibition at 4°, and was greater than that in the presence of 10 mM Na^+.

Interaction of phosphorylated Na,K-ATPase and ouabain at 25°. Inhibition at 25° by the ouabain trapping method did not decrease; in contrast to the inhibition at 4°, it even *increased* in the presence of high concentrations of Na^+ (Fig. 4; Table 4). The presence of K^+ in the CDTA-ouabain solution reduced this inhibition, but its effect was decreased with the increase in Na^+ concentration. In contrast to the results at 4°, the addition of ADP to the CDTA-ouabain solution caused a reduction of ouabain binding, and the extent of this reduction was increased with the increase in Na^+ concentration (Table 4). Oligomycin also reduced ouabain inhibition as did ADP (Fig. 4). The dependence of this effect of oligomycin on the Na^+ concentration was shown much more clearly at 25° (Fig. 4) than at 4° (Fig. 1).

The time courses of ouabain binding with phosphoenzyme are shown in Fig. 5. At 25°, the initial rate of ouabain binding with the phosphoenzyme in 10 mM Na^+ was greater than that of the phosphoenzyme in 1000 mM Na^+, even though the level of ouabain binding in 10 mM Na^+ was lower than that in 1000 mM Na^+. When oligomycin (50 μg/ml final concentration) was added to the phosphoenzyme with ouabain at 25°, the binding of oua-

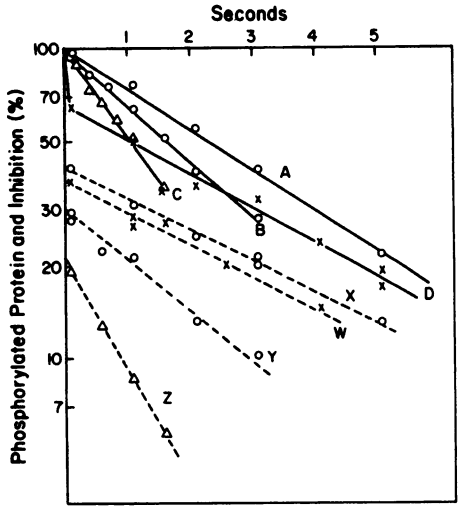


FIG. 2. *Stabilities of phosphoenzyme and active intermediate for ouabain binding*

Phosphorylation of eel enzyme was performed at 10° with 20 mM Na^+, 2 mM Mg^{2+}, 40 mM Tris-HCl buffer (pH 7.6), and 20 μM ATP. After 1-sec phosphorylation, the reaction was terminated by the addition of 0.5 volume of 150 mM CDTA containing 20 mM Na^+ without (*line A*, *line X*) or with 0.1 mM K^+ (*line B*, *line Y*), 0.2 mM K^+ (*line C*, *line Z*), or 0.5 mM ADP (*line D*, *line W*). For experiments of phosphoenzyme stability, [γ-^{32}P]ATP was used and the amount of phosphoprotein was measured at the indicated intervals. Phosphoenzyme stabilities are shown as *solid lines* (*lines A*, *B*, *C*, and *D*). Changes in the active intermediate for ouabain binding, shown as *dashed lines* (*lines X*, *Y*, *Z*, and *W*), were determined as follows: After 1-sec phosphorylation and termination of CDTA as in the determination of phosphoenzyme, 2 mM ouabain was added at the indicated intervals and the interaction of the phosphoenzyme with ouabain was terminated by the addition of 60 mM KCl and 3 mM ADP after 6 sec. After removal of the unbound ouabain from the reaction mixture on a Sephadex column, the inhibition of the enzyme was determined (see details in the text). The percentile inhibition is shown as the function of the period between the termination of phosphorylation and the beginning of the ouabain interaction. The dephosphorylation rate constants and the decay rate constants for the active intermediate are shown in Table 3.

bain to the phosphoenzyme in 1000 mM Na^+ was reduced remarkably, but the binding of ouabain to the phosphoenzyme in 10 mM Na^+ was not changed by oligomycin.

DISCUSSION

From the experimental results shown in this paper, the ouabain trapping method reported here seems to be a useful method for studying the binding of ouabain with

TABLE 3

Dephosphorylation rate constants and decay rate constants of active phosphorylated intermediates for ouabain binding.

The values are calculated from the slopes shown in Fig. 2.

	Ligands during dephosphorylation and decay of the active intermediate			
	Without K^+ or ADP	0.1 mM K^+	0.2 mM K^+	0.5 mM ADP
	sec^{-1}			
Dephosphorylation	0.30	0.41	0.68	0.25
Decay	0.26	0.37	0.80	0.24

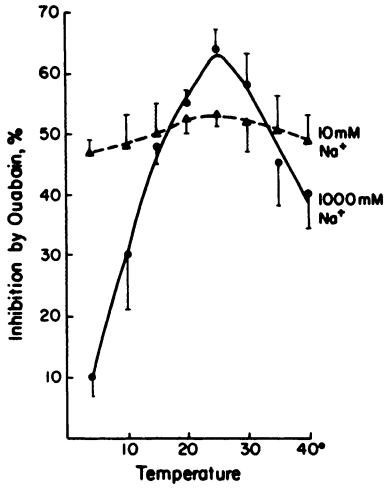


FIG. 3. *Effect of temperature on inhibition by the ouabain-phosphoprotein interaction*

The experimental conditions were similar to those described in Fig. 1 except that the reactions occurred in the presence of 10 mM and 1000 mM Na^+ without oligomycin at various temperatures as indicated.

the active transient intermediate of Na,K-ATPase. This method is especially suitable for studying the effects of reversible reagents such as K^+, ADP, and oligomycin on the active intermediate, because the effects of these reagents are removed by gel filtration. However, this method is applicable only to a Na,K-ATPase preparation which has passed through a Sephadex column without any significant loss of enzyme activity.

In the results shown in Tables 2 and 4, the addition of K^+ to the phosphoenzyme reduced ouabain binding. This finding supports the suggestion by Sen *et al.* (2) that the K^+-sensitive phosphorylated form of Na,K-ATPase, E_2P, is the active intermediate that binds with ouabain. If the phosphorylated active intermediate is a dominant form in the phosphoprotein, the final amount of the inhibition by the present ouabain trapping method is proportional to the amount of the phosphoprotein as described above. As shown in Fig. 2, this was the case when the enzyme was phosphorylated with ATP in the presence of the 20 mM Na^+ at 10°, i.e., when the E_2P was the dominant form and the interconversion between E_2P and E_1P was

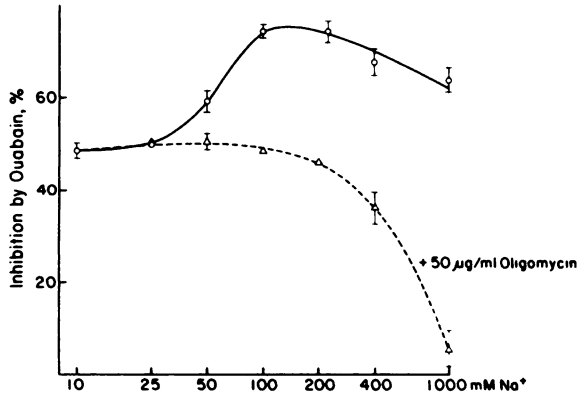


FIG. 4. *Effect of Na^+ concentration on inhibition by ouabain-phosphoprotein interaction at 25°*

The experimental conditions were the same as those described in Fig. 1 except that the temperature was 25°.

TABLE 4

Inhibition by the interaction between phosphoenzyme and ouabain at 25°

Experimental conditions were the same as those described in Table 2 except that the temperature was 25°.

Na^+	ATP during phosphorylation	Inhibition by ouabain		
		Without K^+, without ADP	With 2 mM K^+	With 1 mM ADP
mM	*μM*	%	%	%
10	20	54 ± 2	1 ± 1	45 ± 2
100	20	71 ± 1	7.5 ± 1.5	31 ± 1
1000	100	66 ± 1	54 ± 1 (22 ± 1)[a]	14 ± 1

[a] 5 mM K^+ was used instead of 2 mM.

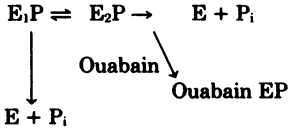
negligible. The stability curves of the phosphoprotein were parallel to decay curves of the active intermediates for ouabain binding in the presence of various K^+ concentrations. This observation strongly confirms the suggestion by Sen *et al.* (2) and also demonstrates the validity of this ouabain trapping method.[2] This parallelism also suggests that the ADP- and K^+-insensitive phosphoenzyme is a minor intermediate for ouabain binding.

On the other hand, the following points might suggest that E_1P could bind with ouabain under some conditions. (a) The eel enzyme used here forms mainly E_1P in the presence of 50–200 mM Na^+, as shown in the preceding paper (8), but the ouabain binding in this range of Na^+ concentration is not less than that in the presence of 10 mM Na^+, in which mainly E_2P forms by the phosphorylation (Figs. 1 and 3; Tables 3 and 4). (b) The presence of ADP in the CDTA-ouabain solution reduced the ouabain inhibition within a certain range of Na^+ concentration (Tables 1, 3, and 4).

These results seem to be explained by the conversion of E_1P to E_2P by ouabain, resulting in the binding of ouabain. This conversion of E_1P to E_2P by ouabain is similar to that with the effect of K^+ (8). The phosphoprotein of Na,K-ATPase formed in the presence of Na^+, Mg^{2+}, and ATP seems to be an equilibrated mixture of E_1P and E_2P, and their ratios are dependent on the Na^+ concentration, as reported by Taniguchi and Post (16).

At 4°, the rate of conversion of E_1P to E_2P and the reverse reaction rate seem to be low. Specifically, when the amount of E_2P is low, ouabain binding by the ouabain trapping method is small, and the addition of ADP does not influence significantly the net ouabain binding. Even though E_1P predominates in eel enzyme in the presence of 50–200 mM Na^+ (8), E_1P can bind with ouabain, but in this case ADP or oligomycin partially prevents this binding. The rate of interconversion of E_1P to E_2P seems to

[2] If the E_1P cannot be ignored, the following reaction system must be considered:



where the formation of ouabain-enzyme complex will not follow the simple parallel first-order kinetics, since the dephosphorylation and binding of E_2P and ouabain are not simple first-order reactions.

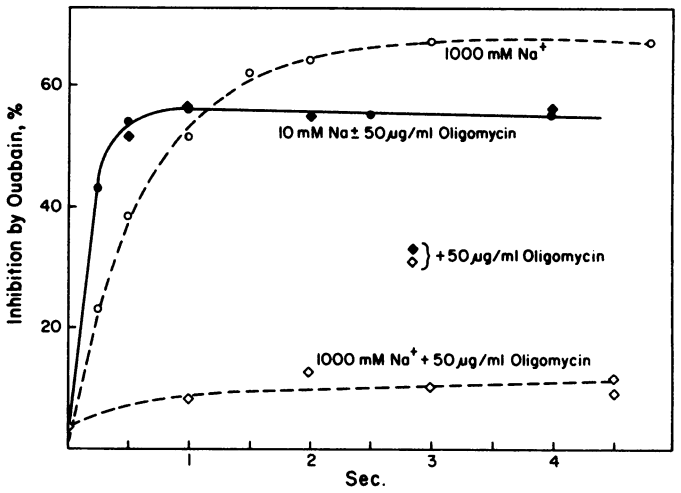


FIG. 5. *Time course of ouabain binding with phosphoenzyme at 25°*

After a 1-sec phosphorylation, as described in Fig. 1, 0.5 volume of a mixture containing 150 mM CDTA, 6 mM ouabain, and the same concentration of Na^+ with or without oligomycin (150 μg/ml) was added. At the indicated intervals, the ouabain-phosphoprotein interaction was terminated by the addition of 60 mM KCl and 3 mM ADP. After removal of the unbound ouabain and oligomycin by gel filtration, inhibition of the enzyme was measured. *Dashed lines* and *open symbols* show the results obtained in the presence of 1000 mM Na^+; *solid lines* and *closed symbols* show those obtained in the presence of 10 mM Na^+. ◇, ◆, Ouabain inhibition in the presence of oligomycin (50 μg/ml); ○, ●, inhibition in the absence of oligomycin.

increase with temperature. As shown in Fig. 2, ouabain binding with the phosphoprotein formed in 1000 mM Na^+, which is believed to be the E_1P-predominant form at any temperature, increased 6 times as a result of the temperature change from 4° to 25°; however, ouabain binding with E_2P changed only slightly. At 25°, the amount of ouabain binding with E_1P was greater than that with E_2P. [The reason why the phosphoprotein in 1000 mM Na^+ can bind more ouabain than that in 10 mM Na^+ may be due to the higher stability of phosphoprotein, as shown in figure 7 of the preceding paper (8)]. Binding of ouabain with E_1P at 25° was reduced remarkably by the addition of ADP (Table 4) and oligomycin (Figs. 4 and 5). These results strongly suggest that ouabain binding with E_2P perturbs the equilibrium of a mixture predominantly consisting of E_1P, and causes the conversion of E_1P to E_2P, creating an increasing amount of ouabain-Na,K-ATPase complex.

REFERENCES

1. Schwartz, A., G. E. Lindenmeyer, and J. C. Allen. The sodium-potassium adenosine triphosphatase: pharmacological, physiological and biochemical aspects. *Pharmacol. Rev.* 27:3–134 (1975).
2. Sen, A. K., T. Tobin, and R. L. Post. A cycle for ouabain inhibition of sodium and potassium dependent adenosine triphosphatase. *J. Biol. Chem.* 244:6596–6604 (1969).
3. Post, R. L., S. Kume, T. Tobin, R. Oreutt, and A. K. Sen. Flexibility of an active center in sodium-plus-potassium adenosine triphosphatase. *J. Gen. Physiol.* 54:306–326 (1969).
4. Siegel, G. J., G. J. Koval, and R. W. Albers. Sodium-potassium activated adenosine triphosphatase. VI. Characterization of the phosphoprotein formed from orthophosphate in the presence of ouabain. *J. Biol. Chem.* 244:3264–3269 (1969).
5. Banerjee, S. P., S. M. Wong, V. K. Khama, and A. K. Sen. Inhibition of sodium- and potassium-dependent adenosine triphosphatase by *N*-ethyl maleimide. I. Effects on sodium-sensitive phosphorylation and potassium sensitive dephosphorylation. *Mol. Pharmacol.* 8:8–17 (1972).

6. Wallick, E. T., B. M. Anner, M. V. Ray, and A. Schwartz. Effect of temperature on phosphorylation and ouabain binding to *N*-ethylmaleimide-treated (Na^+,K^+)-ATPase. *J. Biol. Chem.* **253**:8778–8786 (1978).
7. Post, R. L., G. Toda, and F. N. Rogers. Phosphorylation by inorganic phosphate of sodium plus potassium ion transport adenosine triphosphatase. *J. Biol. Chem.* **250**:691–701 (1975).
8. Yoda, A., and S. Yoda. Formation of ADP-sensitive phosphorylated intermediate in the electric eel Na,K-ATPase preparation. *Mol. Pharmacol.* **22**:693–699 (1982).
9. Yoda, A., and S. Yoda. The interaction of ouabagenin and (Na^+ + K^+)-ATPase in the presence of Na^+, Mg^{2+}, and ATP. *Mol. Pharmacol.* **19**:62–67 (1981).
10. Yoda, A. Association and dissociation rate constants of the complexes between various cardiac monoglycosides and Na,K-ATPase. *Ann. N. Y. Acad. Sci.* **242**:598–616 (1974).
11. Hobbs, A. S., R. W. Albers, and J. P. Froehlich. Potassium-induced changes in phosphorylation and dephosphorylation of (Na^+ + K^+)-ATPase observed in the transient state. *J. Biol. Chem.* **255**:3395–3402 (1980).
12. Mårdh, S., and R. L. Post. Phosphorylation from adenosine triphosphate of sodium- and potassium-activated adenosine triphosphatase. *J. Biol. Chem.* **252**:633–638 (1977).
13. Yoda, A., and S. Yoda. A new simple preparation method for Na,K-ATPase-rich membrane fragments. *Anal. Biochem.* **110**:82–88 (1981).
14. Yoda, A. Structure-activity relationships of cardiotonic steroids for the inhibition of sodium- and potassium-dependent adenosine triphosphatase. I. Dissociation rate constants of various enzyme-cardiac glycoside complexes formed in the presence of magnesium and phosphate. *Mol. Pharmacol.* **9**:51–60 (1973).
15. Böhlen, P., S. Stein, W. Dairman, and S. Udenfriend. Fluorometric assay of proteins in the nanogram range. *Arch. Biochem. Biophys.* **155**:213–220 (1973).
16. Taniguchi, K., and R. L. Post. Synthesis of adenosine triphosphate and exchange between inorganic phosphate and adenosine triphosphate in sodium and potassium ion transport adenosine triphosphatase. *J. Biol. Chem.* **250**:3010–3018 (1975).

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